Echinococcus granulosus: Distribution of Hydatid Fluid Antigens in Tissues of the Larval Stage

I. Localization of the Specific Antigen of Hydatid Fluid (Antigen 5)

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YARZABAL, L., DUPAS, H., BOUT, D., AND CAPRON, A. 1976. *Echinococcus granulosus*: Distribution of hydatid fluid antigens in tissues of the larval stage. I. Localization of the specific antigen of hyatid fluid (antigen 5). *Experimental Parasitology* 40, 391–396. The indirect immunofluorescent test employing a monospecific antiserum has been used to detect the tissue localization of *Echinococcus granulosus* specific antigen "5."

The antigen was revealed in the inner portion of the germinal "membrane" and in the parenchyma of the protoscoleces. In these stages, it was also demonstrated fixed to the walls of some collecting ducts.

It is postulated that the synthesis of the antigen "5" may occur in specialized cells of both the germinal "membrane" and the protoscoleces of the hydatid cysts.

The osmoregulatory system of *E. granulosus* larvae seems to be involved in the transfer of the substance to the cystic cavity.

INDEX DESCRIPTORS: *Echinococcus granulosus*; Cestoda; Helminth; Parasite; Immunology; Antigenicity; Antigens localization; Immunofluorescence test; Hydatidosis.

Numerous substances have been identified in the fluid of the larval stage of *E. granulosus*. Most of them are capable of causing the formation of antibodies, both in naturally parasitized hosts and in laboratory animals experimentally infected or immunized (Biguet *et al.* 1962, Chordi and Kagan 1965, Capron *et al.* 1966). Some of these antigenic substances are produced by the parasite itself, others originate from the host by filtration through the parasitic "membranes" (Kagan and Norman 1963; Norman *et al.* 1964; Varela-Diaz and Coltorti 1972, 1973; Coltorti and Varela-Diaz 1972, 1974).

Among the components of parasitic origin, Capron *et al.* (1966) identified a particularly important one (antigen "5") characterized by a high immunogenicity and a remarkable specificity. Indeed, Capron *et al.* (1966) and Capron *et al.* (1970) have shown that this antigen: (i) stimulates the early production of antibodies in experimental hydatidosis and in animals undergoing immunization, (ii) is revealed through immunoelectrophoretic analysis in most of the human sera taken from surgically confirmed cases of hydatidosis, and (iii) it is not found in quantities capable of being detected by the immunoelectrophoresis test in antigenic extracts of other helminths, including *E. multilocularis*.

Recently, Bout *et al.* (1974) showed that the substance is a lipoprotein supporting α- and β-carboxyl-esterase enzymatic activities, and possesses a molecular weight of

approximately 60,000. They also revealed that antigen "5" corresponds to antigen "A" of Oriol *et al* (1971) confirming that its immunological properties resist the action of an acid acetate buffer (pH 5.0, 0.005 *M*) but are destroyed by heating to 100 C, or to 70 C in the presence of 0.1 *M* HCl.

The development by Bout *et al.* (1974) of an experimental procedure to obtain monospecific antisera against antigen "5" makes it now possible to determine the sites of distribution of the lipoprotein in the parasite.

This study has been planned to determine the distribution of this antigen at the level of the different tissue components of the larval stage of *Echinococcus granulosus* using a monospecific anti-antigen "5" serum and an indirect immunofluorescence procedure.

Materials and Methods

Antigenic Material

Healthy hepatic hydatid cysts were recovered from naturally parasitized horses sacrificed at Lille slaughterhouse. Hydatid fluid was carefully aspirated and allowed to sediment. The supernatant was centrifuged, dialyzed against 100-vol of distilled water and lyophilized (Capron *et al.* 1966) for use as whole hydatid fluid antigen.

The sediment, containing protoscoleces and brood capsules, was washed in several changes of Hanks' solution. Laminated layer and germinal "membrane" were removed in a single block with a part of the host's reactional layer, cut into small pieces (5 × 5 mm), and washed in three changes of Hanks' solution.

Histological Preparations

The material obtained from hydatid cysts was fixed in Bouin–Hollande Sublimé (HCHO, 2.5 ml; H_gCl_2, 0.7 g; $(CH_3COO)_2$ Cu 2.0g; $(NO)_2$ C_6H_2OH, 3.3 g; and H_2O 100 ml) solution, dehydrated in alcohol at increasing concentrations, treated with butyl alcohol, and embedded in paraffin.

The blocks were cut in 5–6 μm thick sections and mounted on microscope slides.

Sera Used

Rabbit anti-antigen "5" serum. This was prepared in accordance with the procedure developed by Bout *et al.* (1974). The precipitation line, consisting of the chosen antigen combined with rabbit antibody (band 5), was identified in a bidimensional immunoelectrophoretic slide performed with whole hydatid fluid and rabbit anti-whole hydatid fluid serum. After 48 hr of washing with phosphate-buffered saline, pH 7.2 (PBS), the zone of agar containing band 5 was excised. This agar was then broken up in 0.5 ml of saline, and emulsified in 0.5 ml of Freund's complete adjuvant. The antigen–antibody adjuvant mixture was injected into the dermis of one rabbit according to the method of Vaitukaitis *et al.* (1971). Blood samples were obtained 15 days later. The monospecificity of the serum was established by double diffusion, immunoelectrophoresis and two-dimensional immunoelectrophoresis.

Rabbit anti-whole hydatid fluid serum. This was obtained by the technique advocated by Capron *et al.* (1966). Adult rabbits, weighing approximately 3 kg each, were infected subcutaneously, at weekly intervals, with 1 ml of a homogenate composed of 4 mg of lyophilized whole hydatid fluid diluted in 0.5 ml saline, and an equal volume of complete Freund's adjuvant. Hyperimmunization was generally obtained at the tenth week.

Fluorescent conjugate of sheep anti-rabbit immunoglobulin serum. The commercial preparation made by the Institut Pasteur (Paris) was used.

Normal rabbit serum. This was collected from an apparently healthy nonimmunized rabbit kept in the laboratory for 1 month before extract'ng its blood.

Normal horse serum. This came from a clinically healthy, nonimmunized horse.

Absorption of Sera

The rabbit antisera to antigen "5" and whole hydatid fluid, like the rabbit normal serum, were absorbed with normal horse serum (20 mg/ml) to remove artificial or natural antibody activity against host serum antigens.

The fluorescent conjugate of sheep anti-rabbit immunoglobulin serum was absorbed with whole hydatid fluid (10 mg/ml) to eliminate the interaction of antibodies cross-reacting with hydatid antigens present in the sheep serum as a consequence of a naturally acquired cestode infection. Aliquot portions of the rabbit antisera to antigen "5" and whole hydatid fluid were also absorbed with whole hydatid fluid to ascertain that the reactions were specific.

Immunofluorescent Reaction

The slide preparations were deparaffinized, washed in PBS, pH 7.2, and incubated for 30 min at 37 C with rabbit anti-antigen "5" serum or rabbit anti-whole hydatid fluid serum at the optimal dilution.

The preparations were washed with PBS, pH 7.2, and incubated at 37 C for 30 min with the sheep anti-rabbit IgG conjugate.

Evans blue at 1/10,000 was used as counterstain.

The controls were: (i) normal rabbit serum absorbed with normal horse serum and (ii) antisera to antigen "5" and to whole hydatid fluid, absorbed with whole hydatid fluid.

In all positive cases the same section stained with haematoxylin and eosin were examined under an optical microscope in order to identify the reactive areas.

RESULTS

The antibodies of rabbit anti-antigen "5" serum were fixed on the internal edge of the germinal "membrane" (Fig. 1A) and on elements of the parenchyma of the protoscoleces (Fig. 1B). Some of the antibodies of the rabbit anti-whole hydatid fluid serum were distributed in the same areas revealed by the anti-antigen "5" immune serum, but others impregnated (i) the rest of the surface of the germinal "membrane" (Fig. 1C), (ii) the matrix of the tegument of the protoscoleces (Fig. 1D), arrow) and (iii) the wall and the debris of the brood capsule (Fig. 2).

In some sections incubated either with rabbit anti-antigen "5" serum or with rabbit anti-whole hydatid fluid, it was possible to observe a strong and specific fluorescence of the circular structures arranged within the protoscolex parenchyma (Fig. 1B, arrow). A study of the same sections under the optical microscope made it possible to establish that these structures corresponded to the walls of collecting ducts.

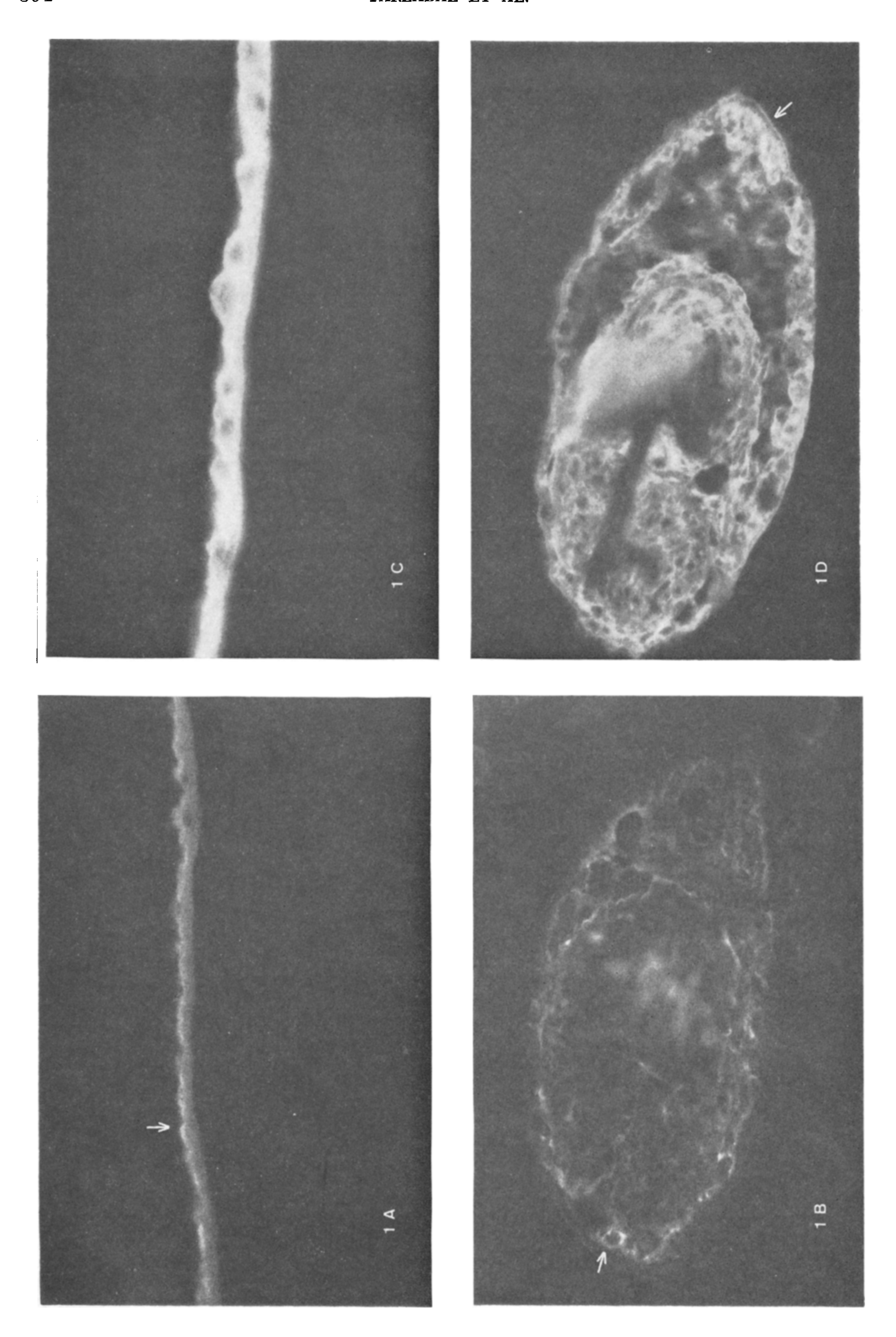
Nonabsorbed rabbit anti-whole hydatid fluid showed antibody activity against antigens located in the laminated and host reactional layers of the cysts. This activity was completely removed with normal horse serum. In the laminated layer these host antigens were restricted to the concentric lines.

The antisera and conjugate controls were always nonreactive.

DISCUSSION

In the present study we used an immune monospecific serum to determine the localization of antigen "5" in the tissues of the larval stage of *E. granulosus*. Using the indirect immunofluorescence technique it was possible to detect the antigen in the inner portion of the germinal "membrane" and in the parenchyma of the protoscoleces of horse hepatic cysts. We were able to establish clearly that the antigen concerned did not exist in quantities detectable by the present method in the other components of *E. granulosus.*

These findings suggest that the synthesis of antigen "5" might take place (i) at the level of the nucleated cells of the innermost sector of the germinal "membrane" and (ii) in some cells of the parenchyma of the


1C
1D
1A
1B

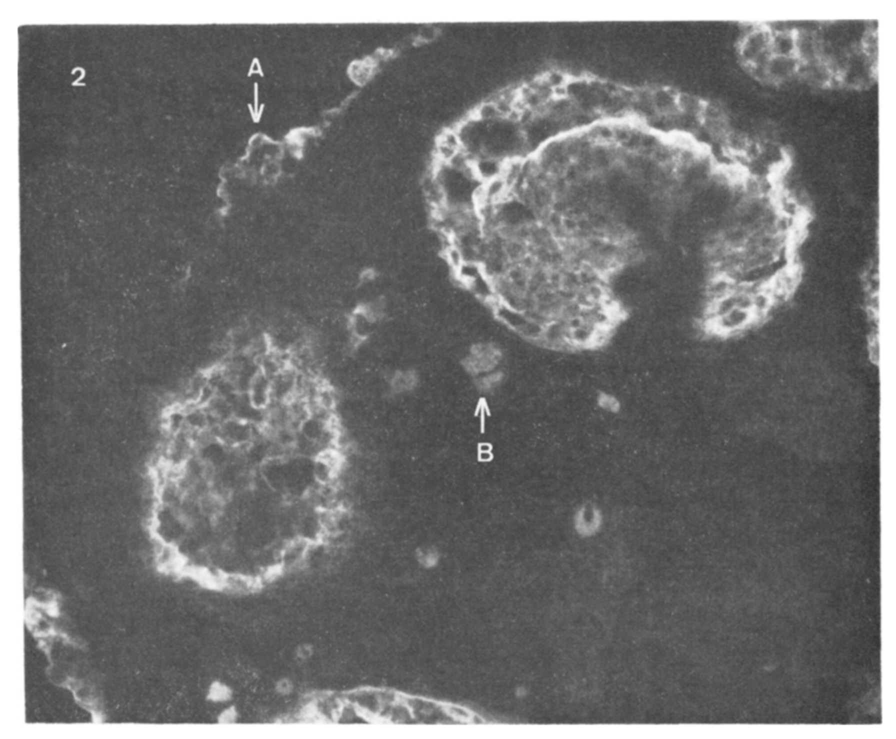


FIG. 2. Section of a brood capsule of horse hydatid cyst of *Echinococcus granulosus* showing the distribution of the antibodies against whole hydatid fluid (640×). (A) Brood capsule wall. (B) Debris from degenerated protoscoleces.

protoscoleces. Our observation of the possible participation of the excretory system in the transport of antigen "5" (and perhaps other parasite antigens as well) permits a further interpretation of the demonstration by Shults and Ismagilova (1962), that antigen–antibody precipitates are formed around the excretory orifices of protoscoleces during incubation in immune sera. Our findings support the suggestion that these precipitates were produced by the interaction of antibodies in the immune sera with antigens synthetized by the protoscoleces and eliminated by the excretory duct.

The laminated "membrane" and the PAS positive layer covering the protoscoleces did not contain antigenic determinants in common with any of the components of the hydatid fluid used in this study.

The absorption test with host serum enabled us to confirm the findings of Coltorti and Varela-Diaz (1974) which showed the existence of proteins of the host on the concentric layers of the laminated "membrane."

The data presented in this paper suggest that our experimental model is a useful approach to the immunochemical investigation of antigen synthesis in *E. granulosus.*

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FIG. 1. Section of horse hydatid cyst of *Echinococcus granulosus* showing the distribution of antibodies against antigenic components of horse hydatid fluid. (A) Anti-antigen "5" antibodies in the inner portion (arrow) of the germinal "membrane" (640×). (B) Anti-antigen "5" antibodies revealing the parenchyma of a protoscolex (the arrow shows a collecting duct) (1000×). (C) Anti-whole hydatid fluid antibodies distributed on the entire surface of the germinal "membrane" (1000×). (D) Anti-whole hydatid fluid antibodies marking the whole surface of a protoscolex, including the matrix of the tegument (arrow) (1000×).

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